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Influence of Temperature, NaCl, and pH on the Growth of *Aeromonas Hydrophila*

ABSTRACT

Growth of clinical isolates of *Aeromonas hydrophila* at various temperatures, pH values, and salt levels was studied in BHI broth. A majority of the isolates grew at 4–5°C and 42°C, and all grew equally well over the range 20–35°C. At 28°C, most isolates could tolerate 4% NaCl, while at 4°C only a limited number grew in 3% NaCl. Similarly, isolates could better tolerate acidic conditions when cultured at 28°C as compared to 4°C. These data suggest that it is likely that *A. hydrophila* strains associated with human gastroenteritis are capable of growing in foods at refrigeration temperatures currently considered adequate for preventing the growth of foodborne pathogens.

INTRODUCTION

AEROMONAS HYDROPHILA is well known as a cause of disease in fish and reptiles (Hazen et al., 1978; Mittal et al., 1980; Shotts et al., 1972) and in recent years, it has been isolated with increasing frequency from cases of diarrhea in humans (von Graevenitz and Bucher, 1983; Goodwin et al., 1983). This bacterium can be readily isolated, often in considerable numbers, from various foods of animal origin, including beef (Ayres, 1960; Jay, 1967), pork (Blickstad and Molin, 1983; Enfors et al., 1979; Myers et al., 1982), raw milk (Kielwein et al., 1969; Kleeberger, 1975), and poultry (Nagel et al., 1960), as well as fish (Molin and Stenstrom, 1984; Boulander et al., 1977), crabs (Faghri et al., 1984), and water (LeChevallier et al., 1980, 1982; Biamon and Hazen, 1983; Rippey and Cabelli, 1979). In contrast to much of the literature in which this organism is considered an undesirable part of the microflora of a food, Buttiaux (1959) has reported that *Aeromonas* plays an important role in the biological processes involved in the manufacture of a French raw sausage ('saucisson cru'). The presence of *A. hydrophila* in a food at spoilage suggests that the organism is capable of competitive growth at refrigeration temperatures (5°C). The topic of *A. hydrophila* as a foodborne pathogen has recently been reviewed (Buchanan, 1984; Buchanan and Palumbo, 1985). The possibility that this microorganism may represent a potential psychrotrophic foodborne pathogen stimulated our interest in determining if clinical isolates of *A. hydrophila* are capable of growth under conditions that would occur in foods. The present study reports on an initial examination of this question, describing the effects of temperature, pH and NaCl content on the growth of *A. hydrophila* in microbiological medium.

MATERIALS & METHODS

THESE STUDIES were done in two series. In the first, the response of five clinical strains to temperatures from 42° to 4°C, NaCl, and pH was measured by plate count, while in the second series, the response of additional clinical strains was measured by optical density measurements.

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Table 1—Source and characteristics of A. hydrophila *strains used in this study*

	Strain	Source	Enterotoxin		Cytotoxin	Hemolysin
			Rabbit	Mouse		
Series I	K140	Asa Ljungh[a]	–	+	+	+
	K144	Asa Ljungh	+	+	+	+
	1653	Asa Ljungh	–	–	+	+
	558	Anna Hostocka[b]	+	–	+	+
	112/70	H. McCarthy[c]	NT[d]	NT	NT	NT
Series II	BA1	Donna Morgan	–	–	+	+
	BA2	Donna Morgan	–	–	–	+
	BA3	Donna Morgan	–	+	–	+
	BA6	Donna Morgan	–	–	+	+
	BA7	Donna Morgan	–	–	–	+
	BA11	Donna Morgan	–	–	–	+
	3647	Donna Morgan	+	+	+	+
	BW37	Donna Morgan	–	–	+	+
	BW83	Donna Morgan	–	–	–	+
	2268	Donna Morgan	–	+	–	+
	C3518A	Donna Morgan	NT	+	+	+
	2815	Donna Morgan	+	+	+	+

[a] Stockholm, Sweden.
[b] Bratislava, CSSR.
[c] Dorset, England.
[d] NT = not tested.

Table 2—Number of days to achieve optical density (A_{600}) of 0.1 at selected temperatures for A. hydrophila *(Series II)*

Strain	Temperature, °C			
	42	28 & 20	12	5
K144	–[a]	1	2	6
BA7	1[b]	1	3	–[c]
1653	–[a]	1	2	6
BA6	1	1	3	–[c]
3647	1	1	3	–[c]
2815	1	1	2	10
BW37	1	1	2	10
BA1	1	1	2	8
BA2	1	1	2	13
BA3	1	1	2	–[c]
BA11	1	1	3	–[c]
BW83	1	1	2	–[c]
2268	1	1	2	13
C3518A	1	1	3	–[c]

[a] No increase in OD after 3 days this temperature.
[b] Time in days to OD of 0.1 or greater.
[c] No increase after 13 days at this temperature.

Cultures

The strains of *Aeromonas hydrophila* used in this study are listed in Table 1 along with their source and characteristics.

Toxin assays

Hemolysin activity was assayed using sheep and rabbit blood agar plates (Remel Media, Houston, TX). Strains were inoculated onto both agar plates and examined for β-hemolysis after overnight incubation at 37°C.

Cytotoxin activity was measured in Y1 adrenal cells (YAC). Cell-free supernatants of *A. hydrophila*, prepared as described previously (Morgan et al., 1983, 1984), were added in 100μL amounts to con-

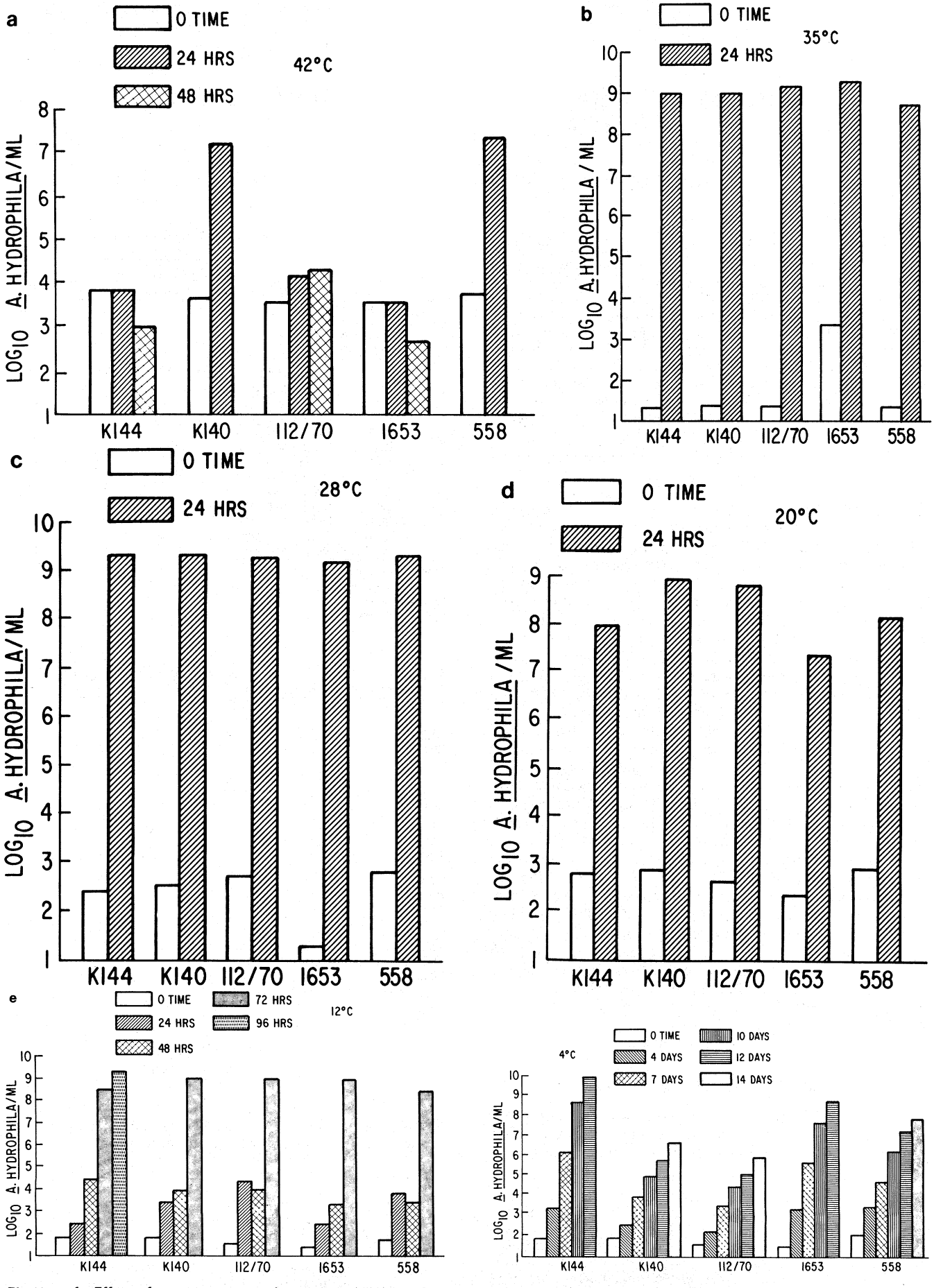

Fig. 1—a-f—Effect of temperature on the growth of strains of A. hydrophila *(Series I cultures): (a.) 42°C; (b.) 35°C; (c.) 28°C; (d.) 20°C; (e.) 12°C; (f.) 4°C.*

tween enterotoxin production by the different strains and their response to temperature, salt, and pH, e.g., compare enterotoxin data from Table 1 with the last column of Table 2. Additional information is needed concerning the possibility that foods may act as a vehicle for this potential human pathogen.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned.

Table 3—Number of days to achieve optical density (A_{600}) of 0.1 at 28° and 4°C and different salt levels for A. hydrophila *(Series II)*

	Salt level in BHI, % (28°C)						Salt level in BHI, % (5°C)			
Strain	0.5	2	3	4	5	6	0.5	2	3	4
2268	1[a]	1	1	1	–[b]	–[b]	29	29	–[c]	–[c]
K140	1	1	2	7	–[b]	–[b]	–[c]	6	10	–[c]
BA2	1	1	1	1	2	–[b]	29	29	–[c]	–[c]
BA7	1	1	1	1	2	–[b]	29	29	–[c]	–[c]
K144	1	1	1	2	–[b]	–[b]	6	6	10	–[c]
558	1	1	2	–[b]	–[b]	–[b]	10	29	–[c]	–[c]
BW37	1	1	1	–[b]	–[b]	–[b]	10	10	–[c]	–[c]
BA3	1	1	1	1	–[b]	–[b]	–[c]	–[c]	–[c]	–[c]

[a] Time in days to OD of 0.2 or greater.
[b] No increase by 11 days at this salt level and temperature.
[c] No difference in OD after 29 days.

Table 4—Number of days to achieve optical density of 0.1 at 28° and 4°C at different pH values for A. hydrophila *(Series II)*

	28°C				5°C			
	pH							
Strain	7.2	6.5	5.5	4.5	7.2	6.5	5.5	4.5
BA1	1[a]	1	1	–[b]	13	17	–[c]	–[c]
3647	1	1	1	–[b]	13	13	17	–[c]
1653	1	1	1	–[b]	6	6	13	–[c]
BA2	1	1	1	3	–[c]	–[c]	–[c]	–[c]
BW37	1	1	1	–[b]	13	13	–[c]	–[c]
BA6	1	1	1	–[b]	6	6	17	–[c]
BA3	1	1	1	3	20	–[c]	–[c]	–[c]
K144	1	1	1	–[b]	6	6	13	–[c]
2268	1	1	1	–[b]	17	20	–[c]	–[c]
K140	1	1	1	–[b]	13	24	–[c]	–[c]

[a] Time in days to OD of 0.1 or greater.
[b] No increase after 20 days.
[c] No increase after 24 days.

and Table 2), but in Series I all grew in 12–14 days to maximum populations, and in Series II, 7 of 14 strains grew.

The data on low temperature growth indicated that, even though all cultures were clinical isolates, most could grow at normal refrigeration temperatures (5°C). Although in Series II, half of the strains did not grow at 5°C, all grew at 12°C. This indicated that only a small temperature increase would allow rapid growth of these strains. Temperature abuse of a food, even for short periods, would permit rapid growth of these organisms. These results are of considerable importance since refrigeration at 5°C is generally considered to be an adequate means of preventing the growth of food poisoning bacteria.

The ability of *A. hydrophila* to grow at low temperatures was reported in the literature (Eddy, 1960). This is also supported by the observation that the organisms are found in refrigerated foods at spoilage and often in considerable numbers (Palumbo et al., 1985; Blickstad and Molin, 1983; Enfors et al., 1979; Nagel et al., 1960; Myers et al., 1982; Jay, 1967; Ayres, 1960).

Salt

After the temperature studies (Series I), *A. hydrophila* K144 was selected for a detailed study of its response to salt and pH because of its vigorous growth at 4°C (Fig. 1f). At 28°C, 4.5% NaCl in the medium delayed growth (Fig. 2); however, at 4°C and 4.5% NaCl, there was a gradual decline. With increasing salt level from 1.5–3.5%, there was an increased lag time and somewhat slower growth.

With Series II strains, the results were similar to those obtained with K144 both at 28°C and 4°C. The data obtained at 28°C (Table 3) permitted determination of maximum salt levels allowing growth: two strains (BA2 and BA7) grew at 5% NaCl at 28°C and two (558 and BW37) did not grow at 4%. Popoff (1984) indicated that motile *Aeromonas* do not grow in nutrient broth containing 5% NaCl. However, in a separate publication, Popoff and Veron (1976) reported that one strain grew at 10% NaCl. As with Series I organisms, the Series II organisms were more sensitive to salt at 5°C. Only two cultures, K140 and K144, grew at 3% and none grew in broth containing 4% NaCl.

Though not studied extensively, some strains of *A. hydrophila* did appear to require a minimum level of salt for growth at low temperatures (4° or 5°C). Strain K140 (Table 3) did not grow in BHI containing only 0.5% NaCl, the basal amount. In Fig. 2, it can be seen that for K144, the culture with 0.5%, growth at 4°C was much slower than the culture with 2.5% NaCl. In a separate experiment with K144 at 4°C, nutrient broth was prepared to contain 0, 0.3, 0.6, 0.9, 1.2, and 1.5% NaCl. The organism grew first at the highest level and then gradually down to 0.6% but not in nutrient broth containing 0 and 0.3% NaCl. Matches and Liston (1972) observed that at 8°C, both *Salmonella heidelberg* and *S. typhimurium* grew in broth with low levels of salt but not in broth without salt. Thus, our observations with *A. hydrophila* appear similar to those reported for other gram-negative rods. Matches and Liston (1972) also reported that salmonellae would grow in the presence of higher levels of salt as the temperature increased.

pH

In Series I (Fig. 3), K144 grew well at 28°C at pH values 7.2 and 6.5. Growth was delayed at pH 5.5 and the viable count declined at pH 4.5. At 4°C, K144 declined at the lower two pH values. The same trend was noted with cultures from Series II (Table 4), with two cultures, BA2 and BA3, growing at pH 4.5 at 28°C. At 4°C, four cultures grew at pH 5.5 while six did not. The apparent difference between Series I and Series II cultures may be due to the length of the incubation period. i.e., 10 days vs 24 days.

The data presented here indicated that clinical isolates of *A. hydrophila* can grow readily under conditions at which food is held. Most of the isolates grew at 42°C, all grew readily at 12°C, and 12 of 19 strains grew at 4–5°C within 2 wk. Under refrigerated storage (5°C), 2 of 8 strains grew at 3% salt within 10 days. At refrigerated temperatures (4–5°C), the organisms tended to be more sensitive to lowering of pH than at higher temperatures. There did not appear to be any relationship be-

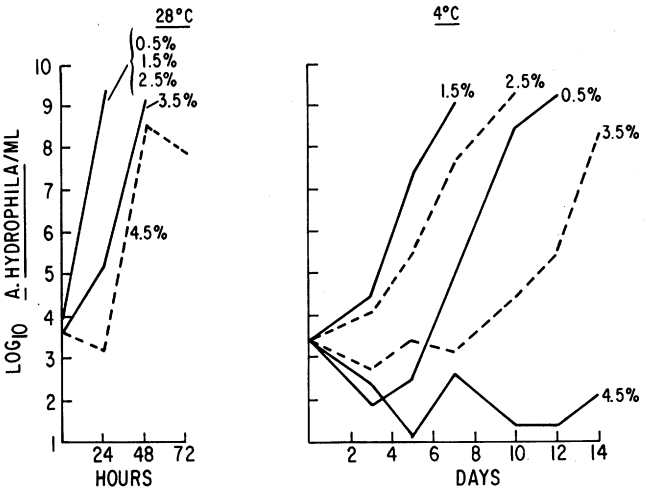

Fig. 2—Effect of salt level in BHI on the growth of A. hydrophila *K144 (Series I) at 28°C and 4°C.*

fluent YAC monolayers. Following an 18–24 hr incubation period at 37°C under 5% CO_2 and 90% relative humidity, the YAC cultures were examined for detachment of the monolayers, which was indicative of lethality. Only supernatants which caused 100% detachment were considered cytotoxic-positive. The overwhelming cytotoxic response observed precluded detection of cytotonic activity (rounding of cell ends).

Enterotoxin activity was measured by suckling mouse (Dean et al., 1972; Morgan et al., 1984) and rabbit ileal loop assays (Evans et al., 1973).

Test medium

Brain Heart Infusion (BHI, Difco) was used as the test medium. The medium as it comes from the manufacturer contains 0.5% NaCl and has a pH of 7.2. It was modified either by the addition of salt (NaCl) or by adjustment of pH (with HCl). In the first series, triplicate 500 mL flasks containing 100 mL of BHI were inoculated with each strain for each variable and were incubated with shaking (200 rpm). In the second series, single BHI (3.5 mL) tube cultures in Spectronic 20 tubes (12 × 100 mm) were used in conjunction with incubation without agitation.

Variables

The effect of temperature, % salt (NaCl) and pH on the growth/survival of the various strains was studied. Temperatures studied were: 42°, 35°, 28°, 20°, 12°, and 4°C. The pH values studied were 7.2 (the pH without adjustment), 6.5, 5.5, and 4.5. The NaCl levels studied were: 0.5% (the salt content of the medium), 1.5, 2.5, 3.5, and 4.5% (g/100 mL) in the first series, and 0.5, 2, 3, 4, 5, 6, 7, and 8% in the second series. The effects of salt and pH were studied at 28° and 4°C.

Effect of variables

In the first series, the effect of the different variables was assessed by viable cell counts surface plated on duplicate nutrient agar (Difco) plates using a Spiral plater (Model DU, Spiral Systems, Bethesda, MD). Dilutions were made in 0.1% peptone (Difco) water. Plates were counted after 24 hr incubation at 28°C except for plates from the pH 4.5 samples for which an additional 24 hr incubation was required for colonies to appear. In the second series, growth was followed by optical density (OD) measurements read at 600 nm in Spectronic 20 spectrophotometer (B & L) against uninoculated tube of the same medium. Readings were made at intervals appropriate to the temperature and other variables being tested. Growth was determined to be positive if the OD of the culture increased 0.1 units. For the OD studies, viable counts were made from the tubes, and the starting counts were ca. 5 × 10^3/mL, making the second series of studies comparable to the first series of studies.

RESULTS & DISCUSSION

Temperature

Although these studies were done in two series and response to the variables (temperature, salt, pH) was assessed by two different methods, the results were complementary and in agreement. The isolates of *A. hydrophila* were able to grow over a temperature range from 42° to 4°C (Fig. 1 and Table 2). In Series I (Fig. 1a), two of the five strains grew at 42°C; the rest declined but not rapidly. In the second series (Table 2), the only strains not growing at 42°C were two included from Series I. A broad temperature band (35° to 20°C) was observed in which all strains grew optimally (Fig. 1b, 1c, 1d, and Table 2). Growth was slower at 12°C (Fig. 1e and Table 2), but maximum populations were reached in 72–96 hr (Fig. 1e). Growth was considerably slower at 4°C and 5°C (Fig. 1f

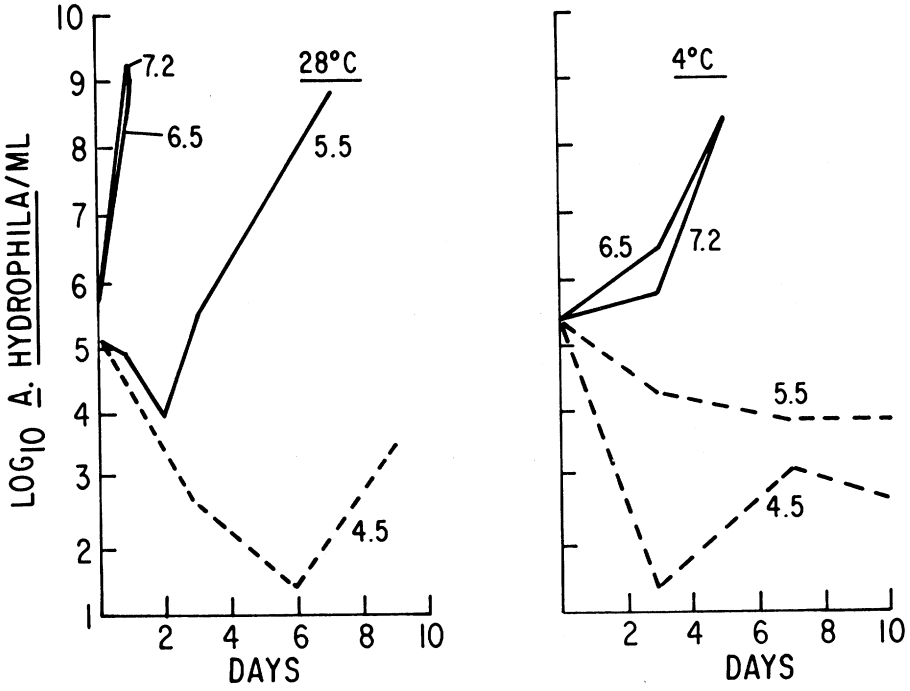

Fig. 3—Effect of pH in BHI broth on the growth of A. hydrophila *K144 (series I) at 28°C and 4°C.*